

REMARKS

Status of the Claims

Claims 1, 5-9, and 13-22 are pending in the present application. Claims 6, 14, and 18-21 are withdrawn as directed to a non-elected invention. Claims 2-4 and 10-12 were previously canceled. Claims 7 and 15 are amended to depend on claims, which are presently being examined. No new matter is entered by way of this amendment. Reconsideration is respectfully requested.

Claim Objections

The Examiner has objected to claims 7 and 15 because of several informalities. In order to overcome this objection, Applicants have amended claims 7 and 15 in order to correct the deficiencies pointed out by the Examiner. Reconsideration and withdrawal of this objection are respectfully requested.

Issues under 35 U.S.C. § 102(a)/(b)

Claims 1, 5, 7-9, 13, 15-17, and 22 are rejected under 35 U.S.C. § 102(a) as allegedly anticipated by Morgan *et al.*, *The Journal of Immunology*, 2003, 171:3287-3295 ("Morgan"), see Office Action, pages 2-5.

Further, claims 1, 5, 7, 9, 13, 15, 17, and 22 are rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Clay *et al.*, *The Journal of Immunology*, 1999, 163:507-513 ("Clay"), see Office Action, pages 5-7. These rejections are respectfully traversed.

According to the Examiner, Morgan or Clay teach all of the elements of the instant claims. In particular, the Examiner states that CD4+/CD8- cells, which are described in the cited references, "are considered helper T1 cells."

The claimed invention

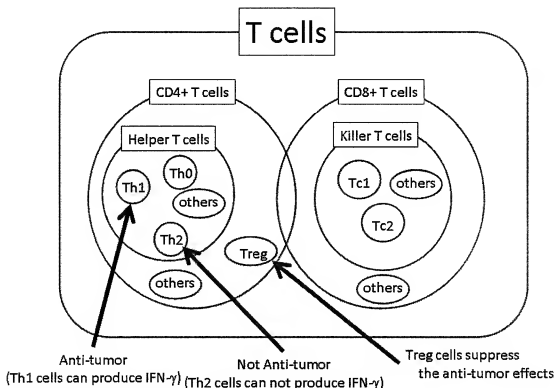
Independent claim 1 is directed to a process of preparing cells for cell therapy, comprising the steps of: inducing helper T1 cells that have a nonspecific antitumor activity isolated from leukocytes isolated from a patient; and imparting antigen specificity to the helper T1 cells, wherein the step of imparting antigen specificity to the helper T1 cells comprises

transducing the helper T1 cells with a MHC class I-restricted T cell receptor gene that recognizes a cancer-associated antigen, and wherein the helper T1 cells are activated or proliferated.

Independent claim 9 is directed to a process of preparing cells for cell therapy, comprising the steps of: inducing helper T1 cells and cytotoxic T1 cells that have a nonspecific antitumor activity isolated from leukocytes isolated from a patient; and imparting antigen specificity to the helper T1 cells and cytotoxic T1 cells wherein the step of imparting antigen specificity to the helper T1 cells and cytotoxic T1 cells comprises transducing the helper T1 cells and the cytotoxic T1 cells with a MHC class I-restricted T cell receptor gene that recognizes a cancer-associated antigen, and wherein the helper T1 cells are activated or proliferated.

The instant claims are not anticipated by the cited references

Applicants submit that neither Morgan nor Clay teach all of the elements of the instant claims. As noted above, the instant claims specify helper T cells. The Examiner's statement that "CD4+/CD8- cells are considered helper T1 cells" is based upon the Examiner's misunderstanding of the biology of T cells. As indicated in the May 12, 2011, response to the January 13, 2011, Final Office Action, a CD4+ cell is not the same as a Th1 (helper T1) cell. Further, a Tc1 cell is completely different from a helper cell. Applicants are resubmitting the Figure below, which depicts the general knowledge in the art regarding T cells.



As shown in the above figure, it was well established in the art that "CD4+/CD8- cells" include "Th0," "Th2," "Treg," and other types of cells. Accordingly, CD4+/CD8- cells do not refer exclusively to Th1 (helper T1) cells.

Those of ordinary skill in the art, having the general knowledge described above, would understand that Morgan and Clay use primary human lymphocytes and peripheral blood lymphocyte cells for introducing an isolated DNA fragment encoding the alpha- and beta-chain of TCR. This teaching of Morgan and Clay is acknowledged by the Examiner, *see Office Action*, page 4, lines 1 and 13, and page 6, line 3.

The lymphocytes described in Morgan and Clay comprise a mixed population of various cells as shown in the figure, above. In contrast, the claimed methods comprise introducing a Class-I restricted TCR gene into *isolated* helper T1 cells. Although peripheral blood cells may include helper T1 cells, those of ordinary skill in the art well-recognize that "isolated helper T1 cells" does not mean "peripheral blood cells" which include various, different cells.

Accordingly, Morgan or Clay do not describe all of the elements of the instant claims. In view of the foregoing, reconsideration and withdrawal of these rejections are respectfully requested.

Issues under 35 U.S.C. §103(a)

Claims 1, 7-9, 15, and 16 are rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Clay in view of U.S. Patent No. 6,610,542 to Bell *et al.*, (“Bell”), see Office Action, pages 7-9. This rejection is respectfully traversed.

As stated above, Clay fails to teach or suggest all of the elements of the instant claims. In particular, Clay fails to teach or suggest isolated helper T1 cells. Bell fails to remedy the deficiencies of Clay. Accordingly, the claimed methods cannot be achieved with the combination of Clay and Bell.

Applicants note that Clay and Morgan, discussed above under the issues under 35 U.S.C. § 102, each list Dr. Steven A. Rosenberg as a co-author, indicating that the work described in these references involve the same concept, *i.e.*, a TCR gene is introduced into peripheral blood cells for the treatment of cancer.

As shown in the above figure, and explained above, peripheral blood cells contain different immune cells, each having distinct function. When a T cell receptor gene is introduced into the different cells of a peripheral blood cell population at the same time and for the purpose of transforming the cells, complex interactions will occur among the immune cells to be transformed. These complex interactions may lead to difficulty in controlling the reactivity of the cells involved in the immune therapy of cancer.

In order to show the state of the art underlying the present invention as well as Morgan and Clay, Applicants submit herewith two articles written by Dr. Rosenberg, which were published after the filing date of the subject application.

In Morgan *et al.*, *Science*, 2006, 314:126-129, wherein Steven A. Rosenberg is a co-author, a T cell receptor was introduced into peripheral blood cells for cancer regression, see Abstract, lines 3-5. In Ray *et al.*, *Clin. Immunol.*, 2010, 136:338-347, wherein Steven A. Rosenberg is also a co-author, another approach was attempted, wherein TCR is introduced into isolated helper T cells (CD4+ CD25-T cells) aiming to achieve immunotherapy of malignant tumors, including melanoma.

The enclosed references demonstrate that the approach to cancer therapy has changed from one involving immune cells (cell population) of the peripheral blood as disclosed in Morgan and Clay, which were cited against the instant claims, to a new approach involving isolated helper T1 cells, as disclosed in the subject application.

Accordingly, the claimed methods are not the same as the methods disclosed in Morgan or Clay and could not have been conceived or achieved based upon any combination of Morgan, Clay and Bell. Further, the claimed methods could not have been derived from Morgan and Clay considering the knowledge in the art at the time of the invention.

In view of the foregoing, the claimed methods are not rendered obvious from the cited references. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION


In view of the above-described amendments and remarks, Applicants believe the present application is in condition for allowance.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Linda T. Parker, Ph.D., Registration No. 46,046, at the telephone number of the undersigned below to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Director is hereby authorized in this, concurrent, and future replies to charge any fees required during the pendency of the above-identified application or credit any overpayment to Deposit Account No. 02-2448.

Dated: FEB 07 2012

Respectfully submitted,

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Attachment(s)



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Cancer Regression in Patients After Transfer of Genetically Engineered Lymphocytes

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Abstract

Through the adoptive transfer of lymphocytes after host immunodepletion, it is possible to mediate objective cancer regression in human patients with metastatic melanoma. However, the generation of tumor-specific T cells in this mode of immunotherapy is often limiting. Here we report the ability to specifically confer tumor recognition by autologous lymphocytes from peripheral blood by using a retrovirus that encodes a T cell receptor. Adoptive transfer of these transduced cells in 15 patients resulted in durable engraftment at levels exceeding 10% of peripheral blood lymphocytes for at least 2 months after the infusion. We observed high sustained levels of circulating, engineered cells at 1 year after infusion in two patients who both demonstrated objective regression of metastatic melanoma lesions. This study suggests the therapeutic potential of genetically engineered cells for the biologic therapy of cancer.

In the past two decades, fundamental advances in immunology have introduced opportunities for the development of cellular-based therapies for the treatment of cancer (1,2). After ex vivo expansion, transfer, and clonal repopulation in patients who have received lymphodepleting conditioning, autologous tumor-infiltrating lymphocytes (TILs) have been found to mediate objective cancer regression in a measurable proportion of patients with metastatic melanoma (3–5). A limitation of this approach is the requirement that patients have preexisting tumor-reactive cells that can be expanded ex vivo. In addition, in many cancer patients, especially those with cancers other than melanoma, it is difficult to identify these tumor-reactive lymphocytes. To overcome this limitation, we set out to develop an approach to cancer immunotherapy based on the genetic modification of normal peripheral blood lymphocytes (PBLs).

Tumor-associated antigens (TAAs) are recognized by the T cell receptor (TCR) on the T lymphocyte surface, which is composed of the TCR alpha and beta chains (6). The genes encoding the TCR that are specific for a variety of TAA have now been cloned, including the TCR-recognizing MART-1 and gp100 melanoma/melanocyte differentiation antigens, the NY-ESO-1 cancer-testis antigen that is present on many common epithelial cancers, and an epitope from the p53 molecule, which is expressed on the surface of approximately 50% of cancers of common epithelial origin (7–12). In each case, these antigens were detected by the TCR when they were presented as peptides by molecules encoded by the major histocompatibility complex protein human lymphocyte antigen (HLA)-A2. In vitro transcribed

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1129003/DC1

RNA from four TAA-reactive TCRs (recognizing MART-1: 27–35, gp100: 209–217, NY-ESO-1: 157–165, and p53: 264–272) were electroporated into CD8⁺ PBLs, which were then cocultured with peptide-pulsed T2 cells. These transfected cells produced large amounts of interferon- γ (IFN- γ) upon stimulation with their respective peptides (Fig. 1A) and were able to recognize HLA-A2–matched tumors, including melanoma, lung cancer, and breast cancer (table S1). Furthermore, transduction with these TCR-encoding retro-viral vectors converted normal PBLs into cells capable of specifically recognizing and destroying both fresh and cultured cells from multiple common cancers (such as sarcoma and breast, lung, esophagus, and liver cancers) in vitro (9–12).

To investigate the ability of genetically engineered PBLs to recognize and destroy tumor cells in vivo, we transduced PBLs derived from patients with melanoma with the genes encoding the alpha and beta chains of the anti-MART-1 TCR. These genes were cloned from a TIL clone obtained from a cancer patient who demonstrated a near complete regression of metastatic melanoma after adoptive cell transfer (ACT) of TILs (5). A retroviral vector was constructed and optimized to express the MART-1 TCR alpha and beta chains (Fig. 1B) (13). Gene transfer efficiency, assessed by staining for the specific V β 12 protein in this TCR, resulted in expression in 30% of the transduced CD8⁺ cells (Fig. 1C), as compared with ~1% of untransduced control cell cultures (gene transfer was about equally divided between CD4 and CD8 cells). Fifteen percent of the transduced CD8⁺ cells bound the MART-1 peptide-specific HLA-A*0201 tetramer (Fig. 1C and table S2). The TCR-transduced cells were biologically active, as demonstrated by the specific secretion of IFN- γ after coculture with both MART-1 peptide-pulsed cells and HLA-A2 positive melanoma cell lines (Fig. 1D).

To investigate the in vivo efficacy of these MART-1 TCR-engineered T cells, we selected 17 HLA-A*0201 patients with progressive metastatic melanoma (Table 1) for treatment. Cancers in all patients were refractory to previous therapy with interleukin-2 (IL-2). T cell cultures from all 17 patients were biologically reactive, with specific secretion of IFN- γ after coculture with either MART-1 peptide-pulsed T2 cells and/or melanoma cell lines expressing the MART-1 antigen (Fig. 1E). Gene transfer efficiencies measured by staining for V β 12 expression in these lymphocytes ranged from 17 to 67% (42%, mean value) (Table 1 and table S2).

Patients received ACT treatments with MART-1 TCR-transduced autologous PBLs at a time of maximum lymphodepletion (13). Three patients in an initial cohort were treated with cells after an extended culture period of 19 days and had cell doubling times ranging from 8.7 to 11.9 days (Table 1, cohort 1, patients 1, 2a, and 3). In these patients, <10% of the transduced cells persisted across the time points tested during the first 30 days after infusion, and \leq 2% of the cells persisted beyond 50 days (Fig. 2A). These first three patients showed no delay in the progression of disease.

In an effort to administer gene-modified lymphocytes that were in their active growth phase, the culture conditions were modified (13) to limit the ex vivo culture period to between 6 and 9 days after stimulation of cells with antibody to CD3 (Table 1, cohort 2, cell doubling times of \leq 2 days). In another cohort, larger numbers of actively dividing cells for ACT were generated by performing a second rapid expansion protocol (14) after 8 to 9 days (Table 1, cohort 3, cell doubling times from 0.9 to 3.3 days). In contrast to the lack of cell persistence seen in cohort 1 patients (Fig. 2A), patients in cohorts 2 and 3 (Fig. 2, B to D) all exhibited persistence of the transduced cells at >9% at 1 and 4 weeks after treatment (range, 9 to 56%). All eight patients who provided samples >50 days after treatment exhibited cell persistence of >17%, and this level of persistence was durable in seven patients during a >90-day monitoring period. In one patient (patient 14), >60% of circulating lymphocytes were positive for the gene-marked cells (Fig. 2C).

In 14 patient samples tested at one month after ACT, quantitative reverse transcription polymerase chain reaction (RT-PCR) assays revealed the presence of vector-derived RNA, confirming that gene expression continued (table S3). All but one of 15 patients analyzed had increased levels of CD8⁺Vβ12 cells at 1 week after treatment, and the levels of 11 of the 15 patients were higher at 1 month as compared to pretreatment levels (Fig. 2E). Of 13 patients that were examined, all had increased MART-1 tetramer-binding cells after treatment (Fig. 2F), and 11 of 14 had increased numbers of enzyme-linked immunosorbent spot-positive cells (table S4).

There was, however, a discordance between the mean persistence of transduced cells at 1 month in cohorts 2 and 3 measured by PCR (26%) as compared to the measurement of Vβ12-expressing cells (8.1%) and of MART-1 tetramer-binding cells (0.8%). This discordance may in part be due to mispairing of the introduced TCR chains with the endogenous chain, as well as to the different sensitivities of the assays. The reduced expression of the transgene in the persisting cells at ≥1 month may also be a function of the described decrease (15) in the transcription of retrovirally inserted transgenes and the decline in metabolic activity during the conversion of activated cells to memory cells. This decrease in expression of the retroviral transgene would be expected to affect the measurement of tetramers, which relies on the aggregation of multiple receptors, more heavily than the detection of Vβ12 cells directly by antibody staining.

Most important, two patients demonstrated a sustained objective regression of their metastatic melanoma assessed by standard criteria [response evaluation criteria in solid tumors (RECIST)] (16). Patient 4, a 52-year-old male, had previously received treatment with interferon-α (IFN-α), a lymph node dissection, an experimental vaccine, and high-dose IL-2. The patient then developed progressive disease in the liver (4.4- by 3.3-cm mass) and axilla (1.3- by 1.2-cm mass). After treatment with the ACT protocol described above, he experienced complete regression of the axillary mass and an 89% reduction of the liver mass (Fig. 3, A and B), at which time it was removed. He remains clinically disease-free at 21 months after treatment. Patient 14, a 30-year-old male, previously received treatment consisting of a lymph node dissection, IFN-α, and high-dose IL-2. He developed an enlarging 4.0- by 2.5-cm mass in the lung hilum. After ACT treatment, he underwent regression of the hilar mass and is now clinically disease-free 20 months later (Fig. 3, C and D). Thus, two patients with rapidly progressive metastatic melanoma showed full clinical regression of disease after the transfer of genetically engineered autologous PBLs.

In responding patients 4 and 14, the number of gene-marked cells in the circulation (assumed to be 1% of total body lymphocytes) increased by factors of 1400 and 30, respectively, as compared to the number of infusion cells. At 1 year after infusion, both responding patients had sustained high levels (between 20 and 70%) of circulating gene-transduced cells (Fig. 3E). This high level of gene-marked cells was confirmed in patient 4 by limiting dilution T cell cloning of circulating lymphocytes at 1 year after treatment, which revealed that 42% (33 out of 79) of T cell clones contained the transgene as assessed by the PCR assay. These two patients also displayed Vβ12 cells that were detectable by antibody staining between 12 and 16% for >300 days after treatment (Fig. 3F). Patients 4 and 14 were also two of four patients who had >1% of circulating tetramer positive cells detectable for >15 days after cell infusion (Fig. 2F), and these two patients demonstrated anti-TAA reactivity in ex vivo coculture assays (table S5). No toxicities in any patient were attributed to the gene-marked cells. Although the genetically modified transferred cells exhibited decreased expression of the transgene with time in vivo, the functional activity was apparently sustained at a level sufficient to mediate the tumor regression that was seen.

Approaches to increase the expression and function of the transgene are being studied, including the possible use of lentiviral vectors, the use of more powerful promoters specific to T cells, the use of higher-affinity TCRs that can mediate CD8 independent antitumor reactivity in CD4 cells, the further optimization of T cell transduction methods, and the production of higher titer clinical-grade viruses. Approaches to prevent chain mispairing may include modification of the TCR constant regions, the insertion of single-chain receptors (17), or the genetic modification of hematopoietic stem cells (18). Because tumor specificity can be conferred on bulk PBL populations with high efficiency, it may be possible to select sub-populations of PBLs that have distinct anti-tumor qualities. Further genetic modification of PBLs to insert cytokine or tissue-homing molecules may be beneficial. Mouse models predict that increased lymphodepletion, either by the addition of total body irradiation to the preparative regimen or by the administration of a vaccine containing the antigen recognized by the transduced TCR, can also enhance treatment effectiveness (19,20), and these modifications are currently being explored in clinical trials.

In human subjects, normal autologous T lymphocytes, transduced ex vivo with anti-TAA-TCR genes and reinfused in cancer patients, can persist and express the transgene for a prolonged time in vivo and mediate the durable regression of large established tumors. Although the response rate (2 out of 15 patients or 13%) seen in cohorts 2 and 3 is lower than that achieved by the infusion of autologous TILs (50%), this method has potential for use in patients for whom TILs are not available. Engineering PBLs to express high-affinity TCRs recognizing the NY-ESO-1 or p53 antigens (Fig. 1A and table S1) enables the in vitro recognition of TAAs expressed on a variety of common cancers, and the use of these genetically engineered cells for the treatment of patients with common epithelial cancers deserves evaluation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

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21. The authors acknowledge the expert help in the care of these patients provided by the Surgery Branch Immunotherapy Fellows; J. Gea-Banacloche for valuable advice concerning the management of infectious complications; the nurses on the 3NW and Surgical intensive care unit wards in the Clinical Center; and NIH, as well as A. Mixon and S. Farid for fluorescence-activated cell sorting analysis. We also thank K. Cornetta and the National Gene Vector Laboratory for production of the clinical-grade retroviral vector. This work was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH.

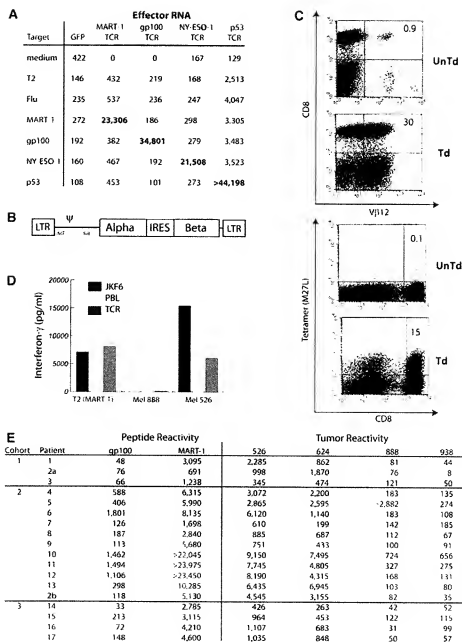
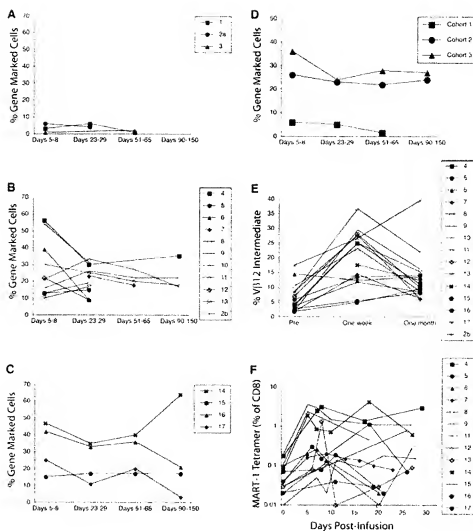


Fig. 1. Transduction and analysis of TCR-engineered cells. (A) CD8⁺ human lymphocytes were electroporated with RNA encoding control [green fluorescent protein (GFP)] or cloned TCRs reactive with HLA-A2 restricted epitopes from the human TAAs MART-1, gp100, NY-ESO-1, and p53. Effector T cells were cocultured with T2 cells pulsed with 1 μ M of the indicated peptide (values are expressed as IFN- γ in pg/ml). Values demonstrating the specific release of cytokine are in bold. (B) Diagram of the recombinant retroviral vector MSGV1AIB used to engineer human lymphocytes. LTR, long terminal repeat; Ψ , extended packaging signal; sd, splice donor; sa, splice acceptor; Alpha, alpha chain; IRES, internal ribosomal entry site; Beta, beta chain. (C) Transduced (Td) lymphocytes were analyzed 5 days after transduction for the

expression of Vβ12 and MART-1 tetramer [Ala²⁷ → Leu²⁷ (A27L)] in CD8⁺ cells in comparison with untransduced (UnTd) cells. Numbers in the upper-right corners indicate the percentage of positive cells in that quadrant. (D) TCR vector-engineered cells from patient 6 (TCR) were cocultured with MART-1 peptide-pulsed T2 cells, HLA-A2⁻ melanoma line (Mel 888), or HLA-A2⁺ melanoma line (Mel 526), and the amount of IFN-γ produced was determined. Control effectors were untransduced cells (PBL) and the MART-1-reactive TIL JKF6 (JKF6). (E) Anti-melanoma properties of genetically engineered lymphocytes were determined for all patients before infusion. The production of IFN-γ (pg/ml) after coculture with peptide-pulsed T2 cells (Peptide Reactivity) and anti-melanoma activity (Tumor Reactivity) for HLA-A2⁺ lines (526 and 624) and HLA-A2⁻ lines (888 and 938).

**Fig. 2.**

Persistence of gene-marked cells. DNA extracted from peripheral blood mononuclear cells (PBMCs) was subjected to real-time quantitative PCR to determine the percentage of vector-transduced cells in patient circulation at various times after infusion. Each line represents data from a separate patient. (A) Cohort 1; (B) Cohort 2; (C) Cohort 3. (D) Mean value of the percentage of gene-marked cells for all patients in each cohort at the given time interval after treatment. (E) The percentage of CD8⁺/Vβ12⁺ cells in the intermediate gate (13) for patients in cohorts 2 and 3 is shown. (F) The percentage of CD8⁺/MART-1⁺ tetramer cells was determined for patients in cohorts 2 and 3 at the times shown. Pretreatment values for each patient are plotted as day 0 after infusion.

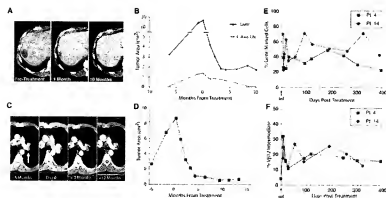


Fig. 3.

Cancer regression in two patients. (A) Computed tomography (CT) images of liver metastasis in patient 4 taken at pretreatment, 1 month, and 10 months after treatment with TCR-engineered T cells. (B) Size of liver and axillary tumors and tempo of regression of tumor sites in patient 4. Day 0, beginning of treatment. L Axill LN, left axillary lymph node. (C) CT images of hilar lymph node metastasis in patient 14; pretreatment, day 0, and 2 months and 12 months after treatment. The white arrow indicates the mass in the lung hilum. (D) Size of tumor and tempo of regression in patient 14. (E) Quantitation of gene-marked cells in the PBMCs of patients 4 and 14 was determined by real-time quantitative PCR. Pt, patient. Day of infusion (Inf.) indicated by arrow. (F) The percentage of CD8⁺/Vβ12⁺ cells in the intermediate gate (13) in the circulation of patients 4 and 14.

Table 1

Patient demographics, treatments received, and clinical outcome. Ln, lymph node; Cu, cutaneous; Sub, subcutaneous; Li, liver; Lu, lung; Ad, adrenal; Pa, pancreas; Br, brain; Hi, hilum. NR, no response; PR, partial response; MR, minor or mixed response.

| Cohort | Patient | Age/ sex | Total cells infused ($\times 10^6$) ^a | CD4/ CD8 ratio (%) | VB12 (%) | MART-1 cells infused ($\times 10^6$) ^b | Days in culture | Doubling time (days) ^c | IL-2 doses ^d | Sites of evaluable disease | Response (duration in months) ^e |
|--------|---------|-------------|---|-----------------------------|-------------|--|-----------------|--------------------------------------|-------------------------|----------------------------------|---|
| 1 | 1 | 28M | 11.0 | 27/73 | 67 | 7.4 | 19 | 8.7 | 7 | Ln, Cu | NR |
| | 2a | 44F | 13.0 | 3/95 | 64 | 8.3 | 19 | 11.9 | 10 | Ln, Cu | NR |
| | 3 | 38M | 14.0 | 17/82 | 35 | 4.9 | 19 | 10.0 | 11 | Cu, Sub | NR |
| | 4 | 52M | 1.0 | 50/50 | 42 | 0.5 | 6 | 1.4 | 9 | Li, Sub | PR(1) |
| | 5 | 48M | 1.0 | 17/82 | 35 | 1.7 | 7 | 1.0 | 7 | Ln, Sub | NR |
| | 6 | 55F | 7.0 | 37/72 | 51 | 3.2 | 7 | 1.0 | 8 | Ln, Sub | NR |
| 2 | 7 | 56M | 9.0 | 75/21 | 40 | 3.6 | 7 | 1.0 | 5 | Ln, Lu | NR |
| | 8 | 37M | 6.1 | 68/40 | 32 | 1.9 | 7 | 1.3 | 12 | Ln, Lu | NR |
| | 9 | 53M | 4.2 | 72/24 | 41 | 1.7 | 7 | 2.0 | 9 | Ln, Ad, Sub | MR |
| | 10 | 53M | 5.4 | 57/43 | 34 | 2.8 | 6 | 0.6 | 5 | Ln, Sub | NR |
| | 11 | 45M | 6.5 | 79/22 | 45 | 2.9 | 6 | 0.7 | 5 | Br, Sub | NR |
| | 12 | 32F | 4.7 | 30/60 | 61 | 3.2 | 6 | 0.9 | 7 | Ln, Sub | NR |
| | 13 | 41M | 7.7 | 40/67 | 42 | 3.2 | 6 | 1.9 | 14 | Ln, Cu | NR |
| | 2b | 44F | 2.1 | 30/59 | 53 | 1.1 | 6 | 0.9 | 5 | Hi | PR(20) |
| | 14 | 53M | 36 | 11/60 | 40 | 34.4 | 18+9 | 0.9 | 5 | Br, Sub | NR |
| | 15 | 51M | 1.2 | 11/22 | 45 | 1.9 | 18+9 | 1.2 | 6 | Ln, Li, Sub | NR |
| | 16 | 25F | 33 | 13/76 | 21 | 6.9 | 18+9 | 1.2 | 2 | Ln, Li, Sub | NR |
| 3 | 17 | 20F | 23 | 17/78 | 30 | 6.9 | 17+8 | 1.1 | 3 | Ln, Lu, Sub | NR |

^a This patient was treated twice; treatments were separated by 7 months.

^b Determined based on cell counts in the 2 days before infusion.

^c Total cells infused multiplied by %Vβ12.

^d 720,000 international units/kg every 8 hours. All patients were previously refractory to treatment with IL-2 alone.

^e Based on RECIST criteria.



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MHC-I restricted Melanoma Antigen Specific TCR Engineered Human CD4⁺ T Cells Exhibit Multifunctional Effector and Helper Responses, *In Vitro*

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Abstract

MHC class I-restricted human melanoma epitope MART-1_{27–35} specific TCR engineered CD4⁺CD25[–] T cells synthesize Th1 type cytokines and exhibit cytolytic effector function upon cognate stimulation. A detailed characterization of such TCR-engineered CD4⁺CD25[–] T cells now reveals that they are multifunctional. For example, they undergo multiple rounds of division, synthesize cytokines (IFN- γ , TNF- α , IL-2, MIP1B), lyse target cells, and “help” the expansion of the MART-1_{27–35} specific CD8⁺ T cells when stimulated by the MART-1_{27–35} peptide pulsed DC. Multiparametric analyses reveal that a single TCR-engineered CD4⁺ T cell can perform as many as five different functions. Nearly 100% MART-1_{27–35} specific TCR expressing CD4⁺ T cells can be generated through retroviral vector-based transduction and one round of *in vitro* stimulation by the peptide pulsed DC. MHC class I-restricted tumor epitope specific TCR-transduced CD4⁺ T cells, therefore, could be useful in immunotherapeutic strategies for melanoma or other human malignancies.

Keywords

Cancer Immunotherapy; TCR; Multi functional CD4 T Cells

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Introduction

A role for CD4⁺ T cells in immune response in tumor immunity, in general, and in tumor immunotherapy, in particular, is widely acknowledged [1;2;3]. However, CD4⁺ T cells recognize epitopes on MHC class II molecules and most solid tumor cells do not express MHC class II molecules. These facts impose considerable difficulty in developing a way to incorporate them in therapeutic designs. As such, how to engage CD4⁺ T cells in tumor immunotherapy has become a critical strategic issue. Lately, the use of CD4⁺ T cells transduced to express the α/β chains of a relevant MHC class I-restricted tumor epitope specific TCR has emerged as a mechanism to achieve that goal [4;5;6;7;8]. That such MHC class-I restricted epitope specific TCR-engineered CD4⁺ T cells recognize the epitope on MHC class-I molecules with or without the requirement of co-receptor (i.e., CD8 molecules) engagement and exhibit effector function has been described by several groups in animal models [4;5] as well as in human systems [9;10;11;12]. We have shown that human CD4⁺CD25⁻ T cells transduced to express the α/β TCR chains specific for the Melan-A/MART-1₂₇₋₃₅ epitope, express type I cytokines and exhibit cytolytic function in a co-receptor-independent fashion [12]. Considering the potential of such tumor epitope specific TCR-engineered CD4⁺ T cells in human tumor immunotherapy, we undertook an extended examination of their biology. Here we show that the MHC class I-restricted MART-1₂₇₋₃₅ epitope specific TCR-transduced CD4⁺CD25⁻ T cells undergo multiple rounds of division and exhibit multifunctional effector function (synthesize IFN- γ , TNF- α , IL-2, mobilize lytic granules and exhibit cytolytic effector function against melanoma targets) in a cognate manner without requiring co-receptor-mediated additional signal. They also amplify the expansion of the MART-1₂₇₋₃₅ epitope specific CD8⁺ T cells in an epitope specific CTL generation assay, *in vitro*.

Materials and Methods

Study population, cell lines and reagents

The study population consisted of HLA-A2-positive healthy donors. The study was approved by Institutional Review Board and written consents were taken from all participants. Separation of CD4⁺CD25⁻, CD8⁺ T cells and culture conditions were described previously [12]. T2 cells- a lymphoblastoid cell line with mutated TAP, was a gift from Peter Cresswell, Department of Immunobiology, Yale School of Medicine, New Haven, Connecticut and the MART-1 negative melanoma line A375 cells engineered to express the MART-1 protein (A375-M) have been described before [13]. The melanoma cell lines PT-M and M-202 were established from two HLA-A2.1 positive melanoma patients. MART-1₂₇₋₃₅ (M1) and MAGE-3₂₇₁₋₂₇₉ (M3) peptides were purchased from NeoMPS (USA).

Retroviral vector construction

MART-1₂₇₋₃₅ epitope specific DMF5 TCR was isolated from a high avidity tumor infiltrating lymphocyte (TIL) clone has been previously described [10]. The PG13 packaging cell line [14] to produce DMF5 retrovirus was cultured in DMEM (Hyclone, USA) supplemented with 10% FBS. The cultures were grown to 70% confluence. Fresh medium was added and the supernatant containing the virus was harvested 16 h later.

Generation of MART-1 TCR transduced CD4⁺CD25⁻ and CD8⁺ T cells

CD4⁺CD25⁻ and CD8⁺ T cells were activated by plate-bound anti-CD3 (5 μ g) and anti-CD28 (1 μ g/ml) antibodies in presence of 100U/ml IL-2. After 48hr, the cells were infected with DMF5 TCR retrovirus containing supernatant in the presence of RetroNectin (Takara, Japan) as per manufacturer protocol. 48hr after infection cells were stained with MART-1

specific tetramer (Beckman Coulter, USA) and analyzed by flow cytometry in FACScalibur (BD Biosciences, USA). The transduced cells were rested in culture for 7–10 days with medium changes and used for functional analyses or used after being frozen in FBS with 10% DMSO. The viability of thawed cells was always in excess of 90% and no significant difference in functional profile was observed with the frozen cells.

CFSE labeling and proliferation assays

CD4⁺CD25⁻ and CD8⁺ T cells transduced with MART-1 specific DMF5-TCR were labeled with 1mM CFSE (Invitrogen, USA) in PBS according to published protocol [15]. Autologous DC were matured in LPS as published [12;13] and pulsed with 50mM of peptide. The CFSE labeled CD4F5 or CD8F5 cells were added to the peptide pulsed DC or tumor cell lines in 1:10 (Target:T cell) ratio. After 4 days, cells were analysed by flow cytometry.

Cytokine secretion assay

T2 cells were pulsed with different dilutions of peptide in complete medium for 30min. 2×10^4 peptide pulsed T2 were incubated overnight (approximately 16h) with 2×10^5 CD4F5 or CD8F5 T cells in 500 μ l final volume in each well of a 48 well plate. The supernatants were collected and cytokines (IL-2, IFN γ , TNF α , IL-10, IL-4 and TGF- β) were measured in ELISA using an ELISA kit (R&D Systems, USA).

Intracellular cytokine staining

Reagents for flow cytometric analysis (anti-CD107a-FITC, anti-IL-2-PerCP-Cy5.5, anti-IFN γ -V450, anti-TNF α -PE-Cy7, anti-MIP-1 β -PE, anti-CD3-APC) were purchased from BD Biosciences (USA) and used as directed by the manufacturer. FoxP3 was stained using anti-FoxP3-APC and FoxP3 staining buffer from Miltenyi Biotec Inc (CA, USA) according to manufacturer protocol. For intracellular cytokine assays, 5×10^5 T cells were incubated with 5×10^4 T2 pulsed with peptides. After 6 hr of incubation, the cells were stained and analyzed with LSR-II flow-cytometer (BD Biosciences). The data were analyzed with FlowJo (TreeStar, USA) and polyfunctionality was assessed with PESTLE and SPICE softwares (provided by Mario Roederer, NIH, Bethesda, MD).

Cytotoxicity assay

1×10^3 melanoma target cells were labeled with ^{51}Cr and cytotoxicity was examined by 4 hr ^{51}Cr release assay [16] in presence of 50 fold excess K-562 as cold target competitors.

Co-culture to assess putative helper function of the TCR-engineered CD4⁺ T cells

Previously published MART-1₂₇₋₃₅ specific *in vitro* CTL generation protocol [16] was used as the basic CTL generation assay to assess helper function of the MART-1₂₇₋₃₅ specific TCR transduced CD4⁺CD25⁻ T cells. Briefly, co-cultures were set up with freshly isolated CD8⁺ T cell and DMF5 TCR engineered CD4⁺CD25⁻ T cells (with mock transduced CD4⁺ T cells as control) against the MART-1₂₇₋₃₅ peptide loaded matured autologous DC. After 8–10 days, the numbers of MART-1₂₇₋₃₅ epitope specific CD8⁺ T cells were determined by tetramer staining.

Results

MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4⁺ T cells proliferate upon cognate stimulation

It is now quite clear that $\alpha\beta$ TCRs that are restricted to MHC class I determinants, when expressed on to CD4⁺ T cells, are functional -- i.e., they send productive signal

[9;12;17;18]. We have also previously shown that human CD4+CD25⁻ T cells engineered to express the MHC class-I-restricted MART-1₂₇₋₃₅ epitope specific TCRs synthesize type 1 cytokines and exhibit cytolytic function [11;12]. Although MHC class I-restricted epitope specific TCRs work on CD4+ T cells, such MHC class I-restricted TCR-engineered CD4+ T cells are yet to be fully characterized especially in the context of their potential usefulness in human tumor immunotherapy. Using a different set of MART-1₂₇₋₃₅ epitope specific TCR, DMF5, with improved transduction efficiency [10], we undertook a more detailed characterization of the MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4+CD25⁻ T cells, *in vitro*. We first examined the robustness of the transduction and the efficiency in generating large numbers of MHC class-I restricted melanoma epitope specific TCR-expressing CD4+CD25⁻ T cells. As shown in Fig.1, a large fraction of CD4+CD25⁻ T cells could be transduced with the DMF5 TCR retroviral vector to express the MART-1₂₇₋₃₅ epitope specific TCR and a substantially larger fraction expressing the MART-1₂₇₋₃₅ epitope specific TCR could be obtained after a single *in vitro* stimulation with the MART-1₂₇₋₃₅ peptide-loaded DC. A nearly homogenous population of MART-1₂₇₋₃₅ epitope specific TCR expressing populations could be obtained after a second stimulation (data not shown). Fig. 2A shows the proliferative potential of the TCR transduced CD4+CD25⁻ T cells in comparison with similarly engineered CD8+ T cells (Fig. 2B) assessed in CFSE dilution assay. As shown, the TCR-engineered CD4+ as well as CD8+ T cells exhibit multiple rounds of division when they encounter the epitope on autologous DC (Figs. 2A & 2B). Of considerable interest, they also undergo multiple rounds of division when stimulated by melanoma cells (Fig. 2C).

MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4+ T cells are multifunctional

We then carried out a more extended functional characterization of DMF5 TCR-engineered CD4+CD25⁻ T cells and found that these TCR-engineered CD4+ T cells are multifunctional (Figs. 3 & 4). Figure 3A shows the cytokine synthetic ability (composite data) of the DMF5 transduced CD4+ and CD8+ T cells from 5 different donors. As shown, they synthesize IFN- γ , TNF- α , IL-2, MIP-1 β . They also expose CD107a (Figs. 3B & 4A) upon cognate stimulation and exhibit cytolytic function (Fig. 4B). Of interest, intracytoplasmic staining revealed that a significant fraction of them exhibit more than one function – a sizeable fraction exhibiting multiple cytokine synthesis as well as exposing CD107a (Fig. 3B & C). Importantly, our analysis showed that a cell that makes IL-2 can also synthesize TNF- α and that both IL-2- and IFN- γ -secreting cells expose CD107a, i.e., LAMP (Fig. 3B). Of further interest, the DMF5 TCR-engineered CD4+ T cells do not express FoxP3 and TGF- β , even when stimulated by the appropriate ligand (Fig 3D). The cytolytic function of the TCR-engineered CD4+CD25⁻ T cells is not precisely comparable to that of the CD8+ T cells against all target cells in chromium release assay (Fig 4B). Nonetheless when taken with their ability to expose CD107a (Fig 4A), as well as to lyse melanoma cells (Fig. 4B), the cytolytic function of the TCR-engineered CD4+ T cells is a distinct bonus. Given that the melanoma epitope specific TCR-engineered CD4+ T cells did not express CD8 co-receptors and as CD8 co-receptor expression by TCR engineered CD4+ T cells have been shown to enhance effector function of MHC class-I TCR-engineered CD4+ T cells [11;19], it is possible to argue that they might be made far better lytic effector cells if they were made to co-express CD8 molecules. Co-expressing CD8 molecules has not been a particularly difficult task [11].

MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4+ T cells provide “help” during the activation/expansion of CD8+ T cells bearing endogenous MART-1₂₇₋₃₅ epitope specific TCR, *in vitro*

Given that CD4+ T cells provide important helper functions to CD8+ T cells in cell-mediated immune responses, we examined if the MART-1₂₇₋₃₅ epitope specific TCR

expressing CD4+CD25⁻ T cells could “help” the CD8⁺ T cells in CTL generation/expansion process. In our years of experience with *in vitro* CTL generation studies in the human melanoma model (i.e., co-culturing CD8⁺ T cells with peptide loaded matured DC), we have seldom found optimum functional activation and good expansion of the epitope specific CD8⁺ T cells in the absence of exogenous cytokines such as IL-2 or IL-15. Accordingly, we addressed whether or not the CD4+CD25⁻ T cells could help the CTL activation/expansion process (CTL burst) in our *in vitro* CTL generation co-cultures in the absence of exogenous IL-2. Figure 5A shows the result of a representative co-culture experiment demonstrating that the expansion of the epitope specific CD8⁺ T cell population was substantially amplified by the DMF5 epitope specific TCR expressing CD4⁺ T cells, when they were stimulated by the MART-1₂₇₋₃₅ peptide in the assay. Figure 5B shows the composite data from 5 separate experiments. The DMF5-transduced CD4⁺ T cells from 5 HLA-A2 positive donors were co-cultured with the untransduced CD8⁺ T cells against peptide pulsed DC. The enhancements of MART-1₂₇₋₃₅ peptide specific CD8⁺ T cell expansion (fold expansion of MART-1₂₇₋₃₅ tetramer positive populations), if any, was then determined by flowcytometry. As can be seen, the number of MART-1₂₇₋₃₅ epitope specific CD8⁺ T cells were considerably higher at all three CD4: CD8 ratios and that the increases were observed only when the co-cultures were performed with the MART-1₂₇₋₃₅ peptide. No enhancement was observed with the mock-transduced CD4⁺ T cells or with the DMF5-transduced CD4⁺ T cells in co-culture with CD8⁺ T cells against the HLA-A2 binding control MAGE-3₂₇₁₋₂₇₉ peptide, M3. Of interest, they expanded the relevant TCR expressing CD8⁺ T cells in the CTL generation cultures without any exogenous cytokine supplementation. While it is possible that IL-2 synthesized by the CD4⁺ T cells provided the help, additional work will be needed to determine the underlying mechanism(s) behind the helper effect of the TCR-transduced CD4⁺ T cells.

Discussion

Active specific immunotherapy have produced remarkable clinical responses in some melanoma patients at times [20;21] and adoptive cell therapy with tumor-reactive tumor infiltrating lymphocytes (TIL) have produced more impressive results in selected patients with metastatic melanoma [22]. Melanoma reactive TIL, however, can be generated in about half of melanoma patients and TIL can only rarely be obtained from patients with other histologic cancer types. New strategies are needed to improve the outcome of these types of therapeutic approaches. Given that CD4⁺ T cells do positively influence the priming phase of CD8⁺ CTL response generation as well as facilitate the CTL memory generation process [23;24;25], it is widely acknowledged that among other strategies that could be useful to improve the results of tumor immunotherapy, figuring out a way that would simultaneously engage CD8⁺ as well as CD4⁺ T cells would be very helpful. Additionally, although the need for “antigen specific” CD4⁺ T cells as “helper” cells is not yet clearly established, it is also believed that a strategy that would engage CD4⁺ helper cells and CD8⁺ CTL recognizing a relevant tumor epitope is likely to be highly effective. In this context, the data presented here are noteworthy.

When collectively taken in the context to the question of how to engage cognate CD4⁺ T cells – along with CD8⁺ T effector cells -- in tumor immunotherapy, several interesting points emerge from the data. First, our data clearly show that a large number of melanoma epitope specific TCR-engineered CD4+CD25⁻ T cells can be obtained through viral vector-based transduction and *in vitro* stimulation and that these MHC class I-restricted TCR transduced CD4+CD25⁻ T cells exhibit essentially all the effector functions that are normally expected from CD8⁺ T cells. Additionally, the data show that they are truly “multifunctional” – i.e., they synthesize multiple cytokines, they are cytolytic, and they provide “help” during CTL burst *in vitro* CTL activation/expansion protocol. Given that the

value of multifunctional effector function [26] is increasingly apparent in HIV immunity [27] as well as in tumor immunity [28], the multifunctional nature of the MHC class I-restricted TCR-engineered CD4+CD25⁻ T cells would be useful in tumor immunotherapy. In this context, the robust proliferative potential of the MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4+ T cells after encountering the epitope on DC or after encountering target cells suggests that they could not only be made to proliferate through traditional DC and peptide-based stimulation – a widely used practice in active specific immunization research – they could also be driven to undergo expansion at tumor sites – a highly desirable goal. Tumor antigen-driven proliferation and functions of CD4+ T cells at tumor sites could have many positive effects. It should be, however, acknowledged that although our past study [12] have shown and present observations also show that the TCR-engineered CD4+ T cells exhibit Th1 type phenotype, that CD4+ T cells engineered to express such MHC class I-restricted TCR (at least, a fraction of them) may have T regulatory (Treg) activities under certain conditions. Additional work will be needed to determine condition(s) that would consistently generate Th1 type CD4+ effector T cells and condition(s) that could lead to the generation of epitope specific Treg cells through TCR transgenesis of CD4+ T cells.

Our data showing that they amplify the burst size of the CD8+ CTL add another dimension to their functional repertoire. Given that a role for CD4+ T cells in the CTL priming and in CTL memory generation is well established [23;24;25], the potential usefulness of such MHC class I-restricted epitope specific CD4+ T cells in active specific immunization can be easily envisioned. We have not addressed if the MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4+ T cells could be made to transition into CD4+ memory T cells themselves or if they could also “help” CD8+ T cells to become memory CTL, the possibility exists that under appropriate conditions (*in vitro* and/or *in vivo*) they could serve such purposes. While additional work will be needed to figure out conditions that would make such MHC class I-restricted and epitope specific TCR-engineered CD4+ T cells to serve such purposes, the successful use of this strategy for the induction of T cell memory response in an animal tumor model [4] and the relationship between multifunctional effector response and clinical benefit observed in a recent peptide and CTLA-4 antagonist-based immunization trial [28] strongly argue for moving these types of MHC class I-restricted tumor epitope specific TCR-engineered CD4+ T cells to the clinic. Although our previous study [12] and this study have been carried out with a single (but two different) set of tumor epitope specific TCRs, the remarkably concordant results do not seem to be a reflection of a particular set of TCR. Of note, MHC class I-restricted α/β TCRs specific for other human tumor-associated epitopes have also been found to function when grafted onto CD4+ T cells [9;12;17;18].

The concept of adoptive immunotherapy with TCR-gene transduced T cells has moved to clinical trial [8]. While it is too early to assess its overall effectiveness, this type of cancer immunotherapeutic approach would have limitations. Virtually all the well recognized reasons underlying failures of T cell-based immunotherapy [29] are likely to frustrate adoptive immunotherapy with TCR-transduced T cells. Nonetheless, it should be pointed out, that most approaches to adoptive immunotherapy or active specific immunotherapy for that matter have, so far, been based on strategies designed to harnessing the effector functions of CD8+ CTL as most human cancer cells express only MHC class I molecule-associated epitopes. Two groups of investigators have recently shown that MHC class II-restricted epitope specific TCR-engineered CD4+ T cells exhibit superb anti-tumor effector responses including cytolytic function and that they could be activated and expanded through immunization, *in vivo*, in animal models [30;31]. The data presented here show a potentially novel way to directing MHC class I associated antigen specific CD4+CD25⁻ T cells – as effector cells and as “helper” cells – to human tumors that do not express MHC class II molecules [32;33]. Accordingly, these types of tumor epitope specific and MHC class I-restricted CD4+CD25⁻ T cells – adoptively transferred in patients followed by

immunization (to expand both CD4⁺ T cells and native CD8⁺ T cells bearing endogenous TCR for the given epitope) or adoptively transferred with similar TCR engineered CD8⁺ T cells followed by immunization – could be novel and valuable strategies in active specific or adoptive T cell-based tumor immunotherapy.

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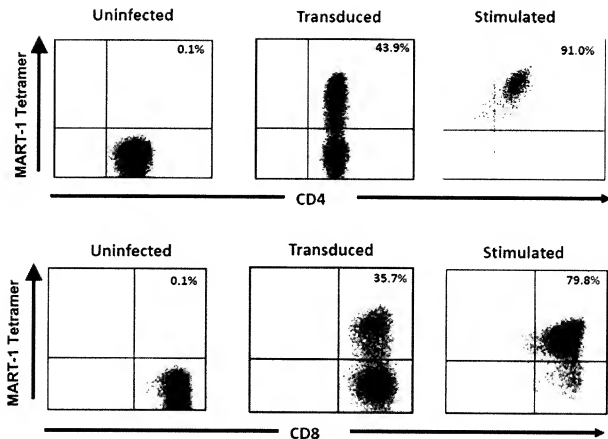


Fig.1.

Transduction of CD4+CD25- and CD8+ T cells with DMF5 TCR expressing retrovirus and further enrichment of the TCR expressing T cells. CD4+CD25- (A) and CD8+ (B) T cells were transduced with the DMF5 retroviral vector, then stimulated by the MART-1₂₇₋₃₅ peptide pulsed autologous DC, and analyzed for MART-1₂₇₋₃₅ epitope specific population by tetramer staining flowcytometry. Representative of six separate experiments is shown.

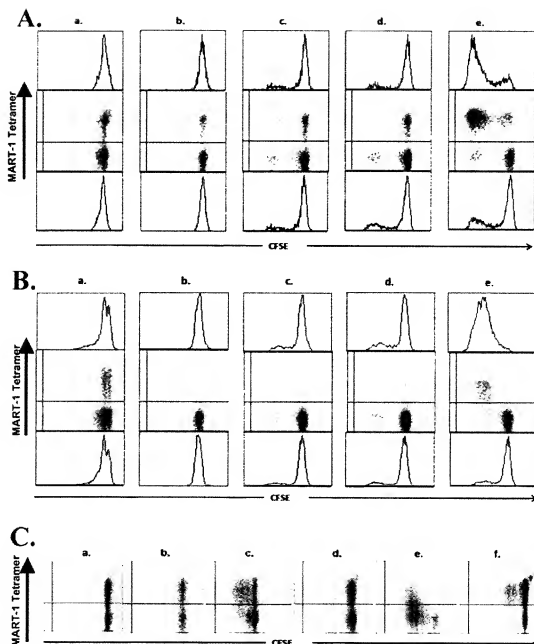


Fig.2.

Proliferative potential of the TCR engineered CD4⁺CD25⁻ and CD8⁺ T. The DMF5 TCR transduced CD4⁺CD25⁻ (A) or CD8⁺ (B) cells were labeled with CFSE (panel a-day 0) and incubated for 4 days alone (panel b), with autologous mature DC (panel c), with DC pulsed with control peptide MAGE-3 (panel d) or with DC pulsed with MART-1₂₇₋₃₅ peptide (panel e). (C). Proliferation of the CFSE labeled DMF5 TCR transduced CD4⁺CD25⁻ T cells when stimulated by melanoma cells. CD4F5 cells at day 0 (a) and at day 4 (b-f) incubated alone (b) or with PT-M (c), wild type A375 (d), A375 cells pulsed with exogenous MART-1 peptide (e), A375M1 (MART-1 transfected A375) (f). Representative of four separate experiments is shown.

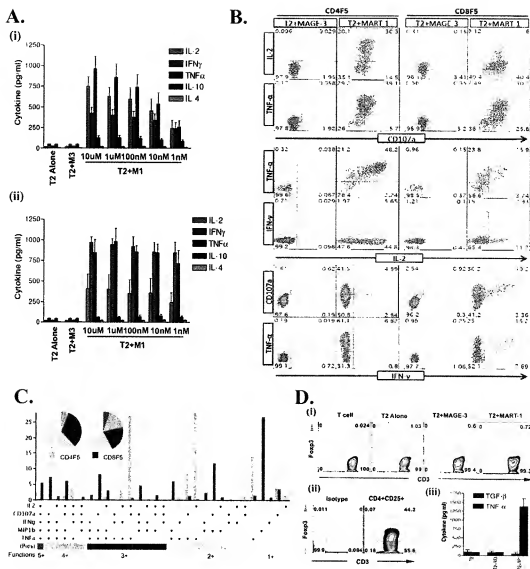


Fig.3.

Functional analyses of the DMF5 TCR transduced T cells. (A). Cytokine synthesis by the DMF5 TCR transduced CD4+CD25- (i) and CD8+ (ii) T cells. The TCR-transduced T cells were stimulated with either the MART-1₂₇₋₃₅ cognate peptide (M1) or MAGE-3₂₇₁₋₂₇₉ control peptide (M3) and cytokine secreted in the supernatant were quantified 16 hr post co-culture set up. Data represents composite analysis of results (mean \pm SEM) of 5 separate experiments with TCR-transduced T cells from 5 different donors. (B). Multiparametric intra-cellular cytokine staining of DMF5 TCR bearing CD4+CD25- (left column) and CD8+ (right column) cells. The TCR-transduced T cells were stimulated with either the MART-1₂₇₋₃₅ cognate peptide (M1) or MAGE-3₂₇₁₋₂₇₉ control peptide (M3) and cells were stained for IL-2, IFN- γ , TNF- α , MIP-1 β and CD107a by intra-cellular staining. Selected combinations are shown as indicated. (C). Analysis of polyfunctional response exhibited by the TCR-transduced CD4+CD25- and CD8+ T cells. The TCR-transduced T cells were stimulated with either the MART-1₂₇₋₃₅ cognate peptide (M1) or MAGE-3₂₇₁₋₂₇₉ control

peptide (M3) and analyzed for multiple functional parameters shown on the X-axis by flowcytometry. The data were analyzed with FlowJo (TreeStar, USA) and polyfunctionality was assessed with PESTLE and SPICE softwares (provided by Mario Roederer, NIH, Bethesda, MD). Each slice of the pie chart shows fraction of total responsive cells were positive for given number of functions (color coded groups below the bar plot). (D). (i) CD4+CD25⁻ TCR transduced T cells were stimulated with MART-1₂₇₋₃₅ cognate peptide (M1) or MAGE-3₂₇₁₋₂₇₉ control peptide (M3) and cells were stained with anti-FoxP3-APC by intracellular staining. (ii) Freshly isolated human peripheral blood CD4+CD25⁺ T cells were stained for FoxP3 as a positive control. (iii) TCR transduced CD4+CD25⁻ T cells were stimulated for 16hr with either T2 alone, M3 or M1 pulsed T2 and supernatant were tested for TGF- β and TNF- α by ELISA. Representative of three separate experiments is shown.

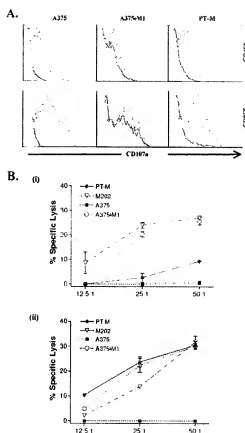
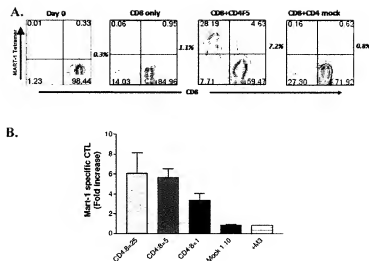


Fig.4.

Cytolytic function of the DMF5 TCR transduced T cells show cytotoxicity against melanoma cells. (A). Staining for surface exposure CD107a by CD4+CD25- (upper) and CD8+ (lower) T cells, when stimulated by A375, MART-127-35 peptide pulsed A375 cells (A375+M1) and the HLA-A2/MART-1 positive line, PT-M. The filled area represents isotype; black line shows interaction with the targets alone; red line shows the interaction with the targets pulsed by the control peptide MAGE-3271-279; and blue line shows the interaction with the targets pulsed with the MART-127-35 peptide. (B). Cytotoxicity by the DMF5 TCR transduced CD4+CD25- (i) and CD8+ (ii) T cells in 4hr ⁵¹Cr release assay (PT-M & M202: naturally MART-1 expressing human melanoma lines, A375: HLA2+/MART-1 neg. line; and A375+M1: MART-127-35 peptide pulsed A375 cells). Representative of three separate experiments is shown.

**Fig.5.**

Amplification of the CD8⁺ CTL expansion by the DMF5 TCR transduced CD4⁺CD25⁻ T cells. (A). A representative experiment is shown where untransduced CD8 T cells were incubated with autologous mature DC pulsed with MART-1 peptide in the presence or absence of CD4F5 T cells without any exogenous cytokine. A representative of four separate experiments is shown. (B). Composite data from similar co-culture experiments with TCR transduced CD4⁺CD25⁻ T cells and untransduced CD8⁺ T cells from 4 different donors. Fold increase of MART-1₂₇₋₃₅ specific population (mean ± SEM) was determined by counting the MART-1 tetramer positive cells in flowcytometry with CD8 gating. Of note, no expansion was observed with the mock-transduced CD4⁺ T cells or with the TCR-transduced CD4⁺CD25⁻ T cells when set up against the MAGE-3₂₇₁₋₂₇₉ control peptide, M3. Results from 4 experiments with 4 different donors are shown.